# Isolation and Properties of the Luciferase Stored in the Ovary of the Scyphozoan Medusa *Periphylla periphylla*

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**Abstract.** Bioluminescence of the medusa *Periphylla* is based on the oxidation of coelenterazine catalyzed by luciferase. *Periphylla* has two types of luciferase: the soluble form luciferase L, which causes the exumbrellar bioluminescence display of the medusa, and the insoluble aggregated form, which is stored as particulate material in the ovary, in an amount over 100 times that of luciferase L. The eggs are especially rich in the insoluble luciferase, which drastically decreases upon fertilization. The insoluble form could be solubilized by 2-mercaptoethanol, yielding a mixture of luciferase oligomers with molecular masses in multiples of approximately 20 kDa. Those having the molecular masses of 20 kDa, 40 kDa, and 80 kDa were isolated and designated, respectively, as luciferase A, luciferase B, and luciferase C. The luminescence activities of *Periphylla* luciferases A, B, and C were $1.2\sim4.1 \times 10^{16}$ photon/mg·s, significantly higher than any coelenterazine luciferase known, and the quantum yields of coelenterazine catalyzed by these luciferases (about 0.30 at 24 °C) are comparable to that catalyzed by *Oplophorus* luciferase (0.34 at 22 °C), which has been considered the most efficient coelenterazine luciferase until now. Luciferase L (32 kDa) could also be split by 2-mercaptoethanol into luciferase A and an accessory protein (approx. 12 kDa), as yet uncharacterized. Luciferases A, B, and C are highly resistant to inactivation: their luminescence activities are only slightly diminished at pH 1 and pH 11 and are enhanced in the presence of $1\sim2$ M guanidine hydrochloride; but they are less stable to heating than luciferase L, which is practically unaffected by boiling.

## Introduction

The bioluminescent deep-sea medusa *Periphylla periphylla* is widely distributed in the oceans. It is especially abundant in certain Norwegian fjords, where large specimens are commonly found—up to 20 cm in diameter, 25 cm in height, and weighing over 600 g (Fosså, 1992). Unlike hydrozoan medusas, which contain calcium-sensitive photoproteins, the glow of the scyphozoan *Periphylla periphylla* is due to a luciferin-luciferase reaction involving coelenterazine (a luciferin) and *Periphylla* luciferase. The luciferase of *Periphylla* occurs as a soluble enzyme and as an insoluble particulate matter (Shimomura and Flood, 1998). The soluble form is found mainly in the exumbrellar epithelia of the dome and lappets and in the dome mesoglea. The particulate matter (about 0.5–1 $\mu$m in size) occurs abundantly in maturing ovarian eggs, and the total amount of luciferase activity in this form per medusa is far greater than the activity of the soluble form. The soluble form, extracted and purified from the lappets, is an unusually heat-stable luciferase, called luciferase L (32 kDa). The highly active particulate matter obtained from the ovary was partially solubilized and extracted with a buffer containing 2 M guanidine hydrochloride, then purified. The enzyme obtained was highly resistant to various denaturants, and it was designated luciferase O (75 kDa).

We recently found that the treatment of the ovarian particulate with 2-mercaptoethanol solubilizes the luciferase and markedly increases its activity. Moreover, we also found that the solubilized luciferase was a mixture of various molecular species having different molecular weights. These and other lines of evidence suggested that the partic-

**Table 1**

*An example of the purification of luciferases A, B, and C from 40 g of ovaries, showing the progress of purification*

| Step | Method | | Total activity ($10^6$ LU) | Specific activity ($10^6$ LU/$A_{280, \, 1cm}$) |
|------|--------|---|---------------------------|-----------------------------------------------|
| 1 | 2-Mercaptoethanol treatment and extraction | | 50 | Not measured |
| 2 | Ether-650 hydrophobic interaction chromatography | | 25 | Not measured |
| 3 | Superdex 200 gel filtration | A | 4.3 | 0.3 |
| | | B | 4.6 | 0.7 |
| | | C | 3.2 | 0.3 |
| 4 | SP-Sepharose anion exchange chromatography | A | 2.6 | 7 |
| | | B | 3.8 | 12 |
| | | C | 2.5 | 25 |
| 5 | SP-650 anion exchange chromatography | A | 1.9 | 21 |
| | | B | 2.8 | 24 |
| | | C | 2.1 | 30 |
| 6 | Superdex 200 gel filtration | A | 1.2 | 13 |
| | | B | 1.6 | 20 |
| | | C | 1.2 | 27 |

1 LU (light unit) corresponds to $5.5 \times 10^8$ photons/s.

ulate material could be the storage form of aggregated luciferase. The present work was undertaken to clarify the chemical nature of luciferase in those aggregates. We have isolated three new molecular species of *Periphylla* luciferase from the ovaries of *Periphylla*, and named them luciferases A, B, and C, respectively. The methods of extraction and purification, and the properties of these luciferases are described and discussed in this paper; we also present a new method of preparing luciferase L.

## Materials and Methods

### Measurement of luminescence activity of luciferase

Luminescence intensity and total light were measured with an integrating photometer model 8020 (Pelagic Electronics, Falmouth, Massachusetts) calibrated with the *Cypridina* bioluminescence reaction (Shimomura and Johnson, 1970). In the assay of luciferase activity, 3 ml of 1 *M* NaCl/0.05% BSA/20 m*M* Tris-HCl (pH 7.8) containing 10 $\mu$l of 0.1 m*M* methanolic coelenterazine was added to a luciferase sample (2~50 $\mu$l) at 24 °C (this is the standard assay condition), and luminescence intensity was measured. Because coelenterazine was present in large excess and luciferase was stable, the rate of the reaction (and thus light emission) was essentially zero-order. One light unit (LU) of luciferase activity on this instrument corresponded to a luminescence intensity of $5.5 \times 10^8$ photons/s. The specific activity of a luciferase sample is defined as "luciferase activity in LU or photons/s, divided by $A_{280 \, nm, \, 1 \, cm}$" except as noted.

### Extraction and purification of luciferase from ovaries

The following is only a general plan of the procedure of purification, because experiments were often modified due to unavoidable variations in the starting materials. At each step of column chromatography, active side fractions were re-chromatographed, and any good fractions recovered were added to the main fractions. In the purification of luciferases A, B, and C (Steps 3-5, below), any side fractions of a target luciferase that contained a different molecular species were combined with a batch of the corresponding luciferase species for further purification. The yields of luciferase at each purification step are shown in Table 1.

*Step 1:* The specimens of *Periphylla* were collected on board R/V *Håkon Mosby* by vertical plankton-net hauls and midwater trawling in Lurefjolden, western Norway. The ovaries and other organs were excised from live specimens, and stored at −75 °C. Frozen ovary (40 g) was thawed and homogenized with a Bamix mixer M122 (Clark National Products, San Dimas, California) in 80 ml of 10 m*M* phosphate buffer (pH 6.6). The homogenate was then centrifuged at 12,000 × g for 10 min at 0 °C. The supernatant was discarded, and the pellets were homogenized in 80 ml of 20 m*M* acetate buffer (pH 5.4) containing 1 *M* KCl and 25 m*M* 2-mercaptoethanol. This mixture was left standing at 0 °C for 3 or 4 h, during which the activity of the sample increased approximately 4-fold. Centrifugation of the mixture gave a clear supernatant (Extract 1). The pellets were mixed with 80 ml of 20 m*M* acetate buffer (pH 4.8) containing 1 *M* KCl and were left standing at 0 °C for 3 or 4 h, then centrifuged to give Extract 2. This extraction was repeated two more times in the same manner, except that the standing time was increased, each time, to 1 day; Extracts 3 and 4 were thus produced.

*Step 2:* Extracts 1 and 2 were combined, and ammonium sulfate was added to make 2.4 *M*. The solution was adsorbed on a column of Toyopearl Ether-650M (Supelco, Bellefonte, PA; 2.5 cm × 7 cm). The column was washed
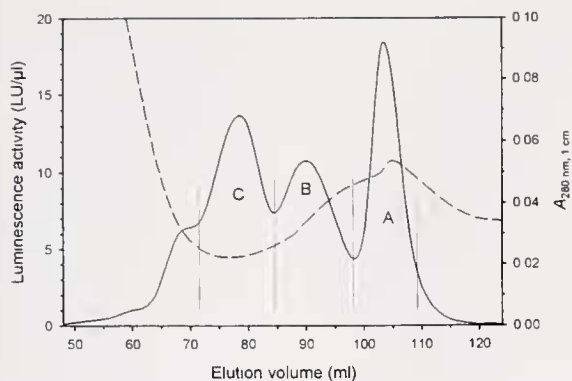
**Figure 1.** An example of the third-step gel filtration on Superdex 200 Prep. Elution curves are shown for luminescence activity (solid line) and the value of $A_{280\ nm,\ 1\ cm}$ (dashed line). A, B, and C are the peaks of luciferases A, B, and C, respectively. The fractions constituting each of these peaks were combined for further purification.

with 2.2 $M$ ammonium sulfate/20 m$M$ acetate buffer (pH 4.8) at room temperature, then luciferase was eluted with 1.8 $M$ ammonium sulfate/20 m$M$ acetate buffer (pH 4.8), and the active fractions were collected. Luciferase fractions that were eluted with ammonium sulfate concentrations lower than 1.8 $M$ were not used in this study. Extracts 3 and 4 were chromatographed on the Ether-650M column in the same manner. All of the active fractions were combined, made up to 2.4 $M$ ammonium sulfate, and then adsorbed on a column of Ether-650M (1.5 cm $\times$ 3.5 cm). The adsorbed luciferase was eluted with 0.5 $M$ KCl/0.01% lauroylcholine chloride (LCC)/20 m$M$ acetate buffer (pH 4.8), giving about 6 ml of concentrated luciferase solution.

*Step 3:* Size-exclusion chromatography was carried out on a column of Superdex 200 Prep (Pharmacia; 1.5 cm $\times$ 72 cm) with 1 $M$ KCl/0.01% LCC/20 m$M$ acetate buffer (pH 4.8) as the eluent. On each run, 3 ml of the sample were injected and the effluent was collected in 2-ml fractions. The fractions were separated into 3 groups—luciferases A, B and C (see Fig. 1)—according to their elution volume, and all the fractions from the same group were combined.

*Step 4:* Cation-exchange chromatography was carried out on a column of SP Sepharose High Performance (Pharmacia; 1 cm $\times$ 6 cm) at room temperature. The eluate from the third step was diluted with two volumes of 0.01% LCC/20 m$M$ acetate buffer (pH 5.5), then adsorbed onto the column. After washing the column with 0.5 $M$ KCl/0.01% LCC/20 m$M$ acetate buffer (pH 5.5), elution was done with a linear gradient of 1.3 $M$ KCl/0.7 $M$ guanidine hydrochloride/0.01% LCC/20 m$M$ acetate buffer (pH 5.5), that increased from 0% to 100% in 23 min.

*Step 5:* Cation-exchange chromatography was repeated with Toyopearl SP-650M (Supelco; 1 cm $\times$ 6 cm). The eluate of the fourth step was diluted with three volumes of 0.01% LCC/20 m$M$ acetate buffer (pH 5.5) and was adsorbed onto the column. The elution was done in the same

manner as in the fourth step. Step 5 effectively eliminated the tailing UV-absorbing impurities that were seen in the fourth step.

*Step 6:* Size-exclusion chromatography was performed on a column of Superdex 200 Prep (1 cm $\times$ 48.5 cm) in 1 $M$ KCl/20 m$M$ acetate buffer (pH 4.8); 1 ml of sample was injected in each run.

*An improved alternative to Steps 1 and 2:* Ovarian tissue (6 g) was briefly homogenized with 20 ml of 10 m$M$ phosphate buffer (pH 6.8), then centrifuged at 20,000 $\times$ $g$ for 10 min, and the supernatant was discarded. The precipitate was mixed with 6 ml of 1 $M$ KCl/1 $M$ guanidine hydrochloride/50 m$M$ acetate buffer (pH 5.4), heated at 80 °C for 1 min, and then centrifuged again. The supernatant, which contained $10^6$ LU of luciferase activity and a large amount of protein, was not used. The precipitate was mixed with 4 ml of 1 $M$ KCl/0.025% BSA/0.3% 2-mercaptoethanol/50 m$M$ acetate buffer (pH 5.4), and left standing at 0 °C for 20 h. Centrifugation of the mixture produced a clear supernatant with a luciferase activity of 2.9 $\times$ $10^6$ LU. Extraction of the precipitate with 1 ml of 1 $M$ KCl/50 m$M$ acetate (pH 5.4) gave an additional luciferase activity of 3.6 $\times$ $10^5$ LU. This alternative method has three advantages: (1) the product obtained has a markedly higher purity than that obtained in Step 2 above; (2) the ratio of luciferases A:B:C can be changed by altering the concentration of 2-mercaptoethanol and the reaction time, because luciferase C progressively dissociates into B and A; and (3) a significant activity loss caused by the use of high concentrations of ammonium sulfate can be avoided.

### A modified method for preparing luciferase L

Only the dome mesoglea (average weight 300 g each), with the thin pigmented layer on the surface removed, were used. The lappets contained greater concentrations of luciferase L, but they were not used because the surface pigment, which drastically decreases the yield of luciferase, is difficult to remove. Cleaned dome mesoglea (500 g) were homogenized in 500 ml of water with 0.3 g of BSA. The homogenate was mixed with 3 teaspoonfuls of Whatman CDR (cell debris remover) and filtered on a Büchner funnel. The filtrate was diluted with two volumes of 10 m$M$ acetate buffer (pH 4.8) and filtered through a column of SP-650M (2.5 cm $\times$ 8 cm). Luciferase adsorbed at the top of the column was eluted with 0.5 $M$ NaCl/0.025% BSA/10 m$M$ acetate buffer (pH 4.8), giving approximately 50 ml of luciferase solution (40,000 LU; this could be safely stored at $-70$ °C, if necessary). The solution was neutralized (pH 7.0) with dibasic sodium phosphate, made up to 2.5 $M$ with ammonium sulfate, and then adsorbed on an Ether-650M column, as described in Step 2 above. The column was washed successively with 15 ml each of 2 $M$ and 1 $M$ ammonium sulfate made with 10 m$M$ phosphate buffer (pH

7.1), and the luciferase L was eluted with 0.5 $M$ ammonium sulfate/acetate buffer (pH 4.8). The material was further purified by chromatography on the columns of SP-650M and Superdex 200, in basically the same manner as reported previously (Shimomura and Flood, 1998). The final yield of purified luciferase L from approximately 5 kg of cleaned domes was 400,000 LU.

### Assay of the luciferase in single eggs, embryos, and juveniles

The specimens were frozen in dry ice on board ship immediately after collection. A single frozen specimen was ground thoroughly in a cold aluminum oxide mortar and pestle with 2 ml of 10 m$M$ phosphate buffer (pH 7.0) containing 0.05% BSA. Fifty microliters of this ground suspension was used to measure the total amount of luciferase activity. The rest of the suspension was centrifuged at 20,000 × $g$ for 10 min, and the amount of soluble luciferase in 50 $\mu$l of the supernatant was then determined. After the supernatant was discarded, the precipitate was mixed with 2 ml of 20 m$M$ acetate buffer (pH 5.5), containing 0.05% each of BSA and LCC (lauroylcholine chloride) 1 $M$ KCl, and 25 m$M$ 2-mercaptoethanol. This extraction/solubilization with 2-mercaptoethanol continued overnight at 0 °C. The total amount of luciferase activity in 50 $\mu$l of this suspension was then measured. After this suspension was centrifuged at 20,000 × $g$ for 10 min, the amount of soluble luciferase in 50 $\mu$l of the supernatant was assayed.

### Results

#### Purification of the three molecular species of Periphylla luciferase from ovary

The insoluble, aggregated form of luciferase was successfully solubilized with 2-mercaptoethanol, presumably by the splitting of disulfide bonds. However, the purification of the solubilized luciferase was difficult, mainly because of certain unusual characteristics of luciferase: its inactivation by dilution—particularly when high concentrations of ammonium sulfate were diluted, its irreversible binding to almost anything, and its expected loss of activity by enzymatic degradation in the early stages of purification.

Because the luciferase bound irreversibly to most chromatographic adsorbents, these materials were unsuitable for purification; indeed their use led to large, often complete, loss of enzyme activity. After extensive tests, a few kinds of adsorbent were found to be relatively safe under certain conditions. At first, BSA was used in the purification of luciferase L to minimize the activity loss, although a protein additive is clearly undesirable in the purification of a protein. However, we have recently found that cationic detergents, such as LCC, effectively prevent the inactivation of luciferase preparations, although the tight binding of the

detergent may cause certain complications by altering the properties of proteins. Guanidine hydrochloride (1-2 $M$) was also highly effective at stabilizing luciferase.

Because of their "sticky" nature, luciferase molecules in crude and partially purified preparations probably exist in complexed forms, bound to some impurities in the solution. Thus, the chromatographic behavior of luciferase in crude preparations may differ from that of pure luciferase, and the behavior of luciferase may change as purification progresses. For example, hydrophobic interaction columns, such as Ether-650M and Butyl Sepharose (Pharmacia), can be used with crude luciferase but not for highly purified luciferase. Similarly, a gel filtration column of Superdex 200 Prep is reliable in most stages of purification, but not with completely pure luciferase.

The purification of the solubilized luciferases from 40 g of ovaries involved over 50 column chromatography runs (summarized in Table 1). In Step 5, the elution curve plotted with luciferase activity and that plotted with $A_{280\ nm,\ 1\ cm}$ value were practically parallel for all three species of luciferase (data not presented), indicating that they were highly pure. The results of Step 6 show decreases in the specific activity, possibly due to two combined effects: the loss of luciferase by adsorption onto the column, and an actual decrease in specific activity; both decreases were caused by the omission of LCC from the buffer used. Assuming that a solution with $A_{280\ nm,\ 1\ cm}$ equal to 1.0 contains 1 mg/ml of luciferase, the yields of purified luciferases at Step 5 (Table 1) are 0.09 mg, 0.12 mg, and 0.07 mg for luciferase A, B, and C, respectively. When Steps 1 and 2 were replaced by the alternative method, the specific activities at Step 4 became 2-3 times higher than those at Step 5 in the original method, although the protein purities remained on comparable levels.

#### Molecular properties of luciferases A, B, and C

The molecular weights of luciferases A, B, and C were estimated by gel filtration on the same Superdex 200 Prep column that was used in Step 6 to purify the luciferases; 0.005% LCC was added to the buffer to minimize adsorption onto the column. The results (Fig. 2) indicated the molecular masses of luciferases A, B, and C to be 19 kDa, 40 kDa, and 80 kDa, respectively. After treating the proteins for 1 day at room temperature with an elution buffer containing 25 m$M$ 2-mercaptoethanol, the main peaks of all the proteins were found at the same position—that corresponding to 19 kDa. This result suggests that luciferases B and C are the dimer and tetramer, respectively, of luciferase A, and that the molecular masses of luciferase A is about 20 kDa, rather than 19 kDa. During the process of purification, three other luciferase species, corresponding to 60 kDa, 120 kDa, and 160 kDa, were observed as relatively minor components. These were not purified.
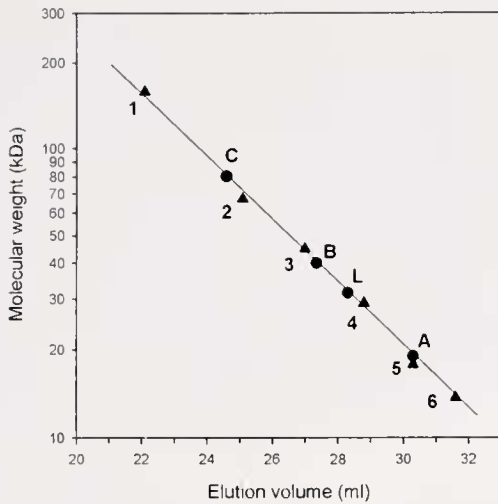
**Figure 2.** Molecular weight estimation of luciferases A, B, C, and L. The gel filtration was carried out on a column of Superdex 200 Prep (1 × 48.5 cm), in 20 m$M$ acetate buffer (pH 4.8), containing 1 $M$ KCl and 0.01% lauroylcholine chloride. Calibration standards: aldolase (1), BSA (2), ovalbumin (3), carbonic anhydrase (4), myoglobin (5), ribonuclease A (6).

SDS-PAGE (polyacrylamide gel electrophoresis) analysis of luciferases A, B, and C under reducing condition (with 2-mercaptoethanol) showed only one major band corresponding to a molecular mass of 24 kDa (Fig. 3); thus, the purity of these proteins and the oligomeric nature of luciferases B and C are verified. Although the molecular mass of the luciferase monomer obtained by gel filtration (19 kDa) does not match well with that obtained by SDS-PAGE (24 kDa), we chose to use the value of 20 kDa for luciferase A (and 40 kDa and 80 kDa for luciferases B and C) as a reasonable approximation, pending the determination of its precise value in the future. We also note here that luciferase L (32 kDa) also yielded luciferase A upon treatment with 2-mercaptoethanol (Fig. 3; see Discussion).

The spectral properties of luciferase A, B, and C were practically identical with those of luciferase L; their absorption and fluorescence spectra indicate that the luciferases are simple proteins, without any chromophore that absorbs or fluoresces in the visible region.

*Enzymatic properties*

The enzymatic properties of luciferases A, B, and C are generally similar to those of luciferase L previously reported (Shimomura and Flood, 1998), but with some notable differences. Thus, the luminescence intensity of luciferase A was highest at 27 °C, and those of luciferases B and C at 30 °C, whereas the luminescence intensity of luciferase L showed no maximum, but steadily increased as the temperature was lowered to almost 0 °C (Fig. 4). A clear difference was also found in their heat stabilities (Fig. 5). Luciferase L is extremely stable to heat, with almost no loss of luminescence activity after

being heated at 95 °C for 2 min. Luciferases A, B, and C are less stable, showing activity losses of 50% or more under the same conditions; the loss seems to be greater with the luciferase species of larger size.

The effects of pH on the luminescence of luciferases A, B, and C were similar to that of luciferase L, showing an optimum at pH 8.0. All *Periphylla* luciferases are highly stable at acidic and alkaline pHs (Fig. 6). The influence of salt concentration on the luminescence activities of luciferases A, B, and C appears essentially the same as that for luciferase L, showing that the optimum salt concentration is about 1 $M$ (Fig. 7). The effect of the concentration of coelenterazine on luminescence intensity is presented in Figure 8, and the Michaelis constants of luciferases A, B, and C calculated from these data are about 0.2 $\mu M$, which is significantly lower than the value for luciferase L (1.1 $\mu M$).

Like luciferase L, luciferases A, B, and C were strongly inhibited by $Cu^{2+}$; but they were not inhibited by thiol agents. Inhibition of luciferase L by thiol agents reported previously (Shimomura and Flood, 1998) must be incorrect because purer preparations showed lesser inhibition; the inhibition seen earlier could have been caused by the activation of thiol-activated proteases.
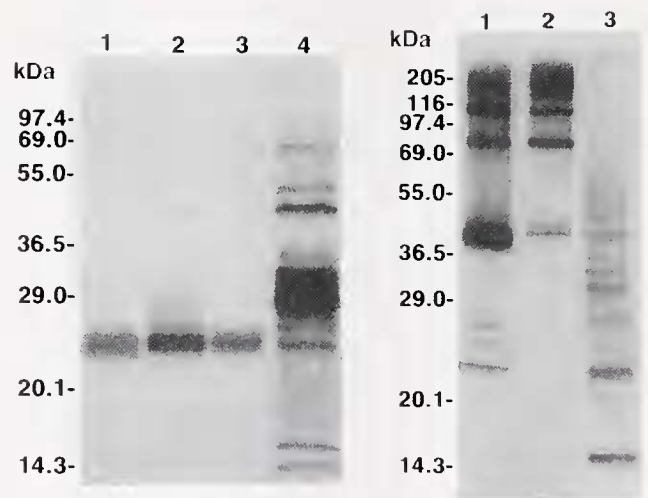


**Figure 3.** SDS-PAGE analysis of luciferases A, B, C, and L. The electrophoresis was carried out under reducing condition for luciferases A, B, C, and L (left panel, lanes 1, 2, 3, and 4, respectively) and under nonreducing condition for luciferases B, C, and L (right panel, lanes 1, 2, and 3, respectively), by the method of Laemmli (1970) using 12% gel; the protein bands were visualized by silver staining. Approximate amounts of protein used: luciferases A, B, and C, 0.3-1.2 $\mu g$; luciferase L, 10 $\mu g$. Marker proteins (not shown): myosin (205 kDa), $\beta$-galactosidase (116 kDa), phosphorylase b (97.4 kDa), BSA (69 kDa), glutamic dehydrogenase (55 kDa), lactic dehydrogenase (36.5 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20.1 kDa), lysozyme (14.3 kDa). Note that the 15-kDa band of lane 4 (left panel) corresponds to the 14.5-kDa band of lane 3 (right panel), in both color and position; the accessory protein of luciferase L is shown as the weak 14-kDa band in lane 4, left panel.
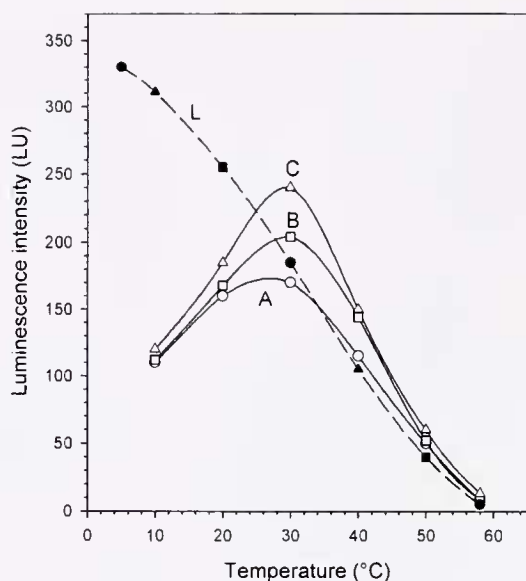
**Figure 4.** Effect of temperature on the luminescence intensities of coelenterazine catalyzed by luciferases A, B, C, and L. The measurements were done in 20 m$M$ Tris-HCl buffer (pH 7.8), containing 1 $M$ NaCl and 0.05% BSA (the standard buffer). The luminescence reaction was started by the addition of 10 $\mu$l of 0.1 m$M$ methanolic coelenterazine. The amount of sample used for measuring each point: luciferase A, 170 LU; luciferase B, 190 LU; luciferase C, 210 LU; luciferase L, 210 LU.

## Luminescence reaction of coelenterazine and its analogs catalyzed by luciferases A, B, and C

The spectra of the luminescence of coelenterazine catalyzed by luciferase A, B, and C were all identical with that of luciferase L, showing a peak at 465 nm. The specific activity (quanta emitted per second, divided by $A_{280 \text{ nm, 1 cm}}$) of the materials obtained in Step 5, Table 1, was 1.21 × $10^{16}$ photons/s for luciferase A, 1.32 × $10^{16}$ photons/s for luciferase B, and 1.65 × $10^{16}$ photons/s for luciferase C, under the standard assay conditions. However, significantly higher specific activities were obtained when the purification included the alternative method for Steps 1 and 2: 3.6 × $10^{16}$ photons/s and 4.1 × $10^{16}$ photons/s for luciferases A and B, respectively (the yield of luciferase C was low). The maximum specific activities obtainable with high concentrations of coelenterazine (over 2 $\mu M$) should be roughly twice these values, based on the data of Figure 8 (note that the coelenterazine concentration in the standard assay is about 0.3 $\mu M$). As a reference to these data, the maximum specific activity of luciferase L reported previously was 8 × $10^{13}$ photons/s (Shimomura and Flood, 1998). The quantum yields of coelenterazine in the luminescence reaction catalyzed at 24 °C by luciferases A, B, and C were 0.287, 0.291, and 0.296, respectively, compared with 0.14 previously reported for luciferase L.

All known coelenterazine luciferases can catalyze the luminescent oxidation of various coelenterazine analogs,

causing luminescence in various intensities—from a negligibly low level to a level several times higher than that of coelenterazine (Inouye and Shimomura, 1997; Nakamura *et al.*, 1997). Using *Periphylla* luciferases A, B, and C, none of more than 20 analogs tested gave a luminescence intensity higher than that of coelenterazine, and only four analogs emitted significant levels of luminescence, each giving the same intensity with the three luciferase oligomers. These four analogs had a substitution at the 2 or 6 position of the imidazopyrazinone ring of coelenterazine, and their relative luminescence intensities, taking coelenterazine as 100%, were: 2-CH$_2$C$_6$H$_5$, 95%; 2-CH$_2$C$_6$H$_{11}$, 21%; 6-C$_6$H$_4$NH$_2$ ($p$), 20%; 6-C$_6$H$_4$NHCH$_3$ ($p$), 31%.

### Luciferase O

Luciferase O (about 75 kDa) had previously been obtained from the ovary by extraction with a buffer solution containing 2 $M$ guanidine hydrochloride (Shimomura and Flood, 1998). On the basis of a chromatographic comparison on a Superdex 200 Prep column, this material was found to be a mixture containing luciferase C (80 kDa) as the main component; the preparation also contained some luciferase oligomers of 60 kDa and 120 kDa and impurity proteins.

## Discussion

### Distribution of luciferase

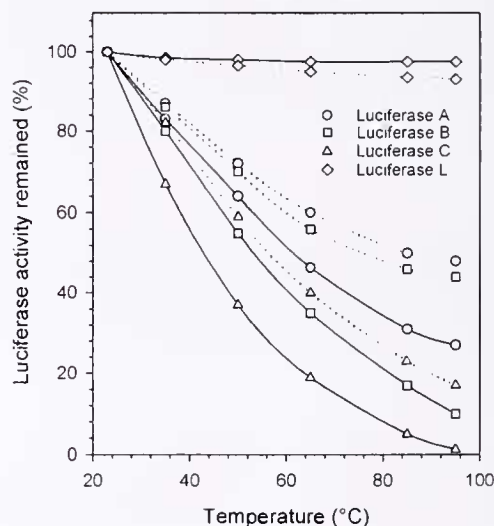*Periphylla* becomes luminescent when coelenterazine is oxidized in the presence of luciferase in certain tissues of



**Figure 5.** Stability of luciferases A, B, C, and L at various temperatures in 20 m$M$ Tris-HCl buffer (pH 7.8) containing 1 $M$ NaCl and 0.05% BSA (solid lines) or 0.01% lauroylcholine chloride (dotted lines). The buffer (1 ml) containing a luciferase sample was added into a glass test tube that had been soaked and pre-equilibrated in a water bath of a desired temperature. After 2 min, the test tube was briefly cooled in cold water, and the luciferase activity in 10 $\mu$l of the sample solution was measured at 24 °C by the standard assay method.
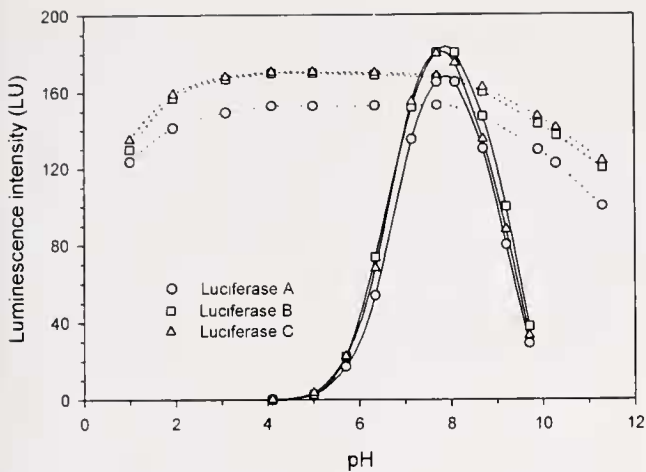
**Figure 6.** Effect of pH on the light intensity of the luminescence of coelenterazine catalyzed by luciferases A, B, and C, and on the stability of the same luciferases. The effect on light intensity (solid lines) was measured in 50 m$M$ phosphate buffers, pH 4.1-7.25, or 50 mM Tris-HCl buffers, pH 7.1-9.7, all containing 1 $M$ NaCl, 0.025% BSA, and 10 $\mu$l of 0.1 m$M$ methanolic coelenterazine. For measuring the effect on stability (dotted lines), luciferase samples were left standing for 30 min at room temperature, in 0.1 ml of a solution having various pH values, then luminescence intensity was measured under the standard condition (pH 7.8). The solutions used were 0.1 $M$ HCl, 0.01 $M$ HCl, 0.01 $M$ acetic acid, 50 m$M$ phosphate, 50 m$M$ Tris-HCl, 0.01 $M$ NH$_3$, and 0.01 $M$ NaOH, all containing 1 $M$ NaCl and 0.025% BSA. The amount of luciferases used for measuring each point were luciferase A, 150 LU; luciferases B and C, 170 LU.

this organism. The luciferase occurs in a soluble form (luciferase L) and also as an insoluble aggregate. The soluble form is responsible for the *in vivo* bioluminescence of the animal and is distributed widely, not only in the epithelial photocytes but also in the mesoglea of the large coronal dome. The insoluble form exists in the particulate matter distributed abundantly in the ovary, particularly in the eggs. The size of the particles, measured by differential filtration, was larger than 0.2 $\mu$m and smaller than 2 $\mu$m; the actual size is probably close to the low end of this range according to previous microscopic observation (Flood *et al.*, 1996). Like soluble luciferase L, the particulate matter is highly active in catalyzing the luminescence of coelenterazine, but its involvement in the *in vivo* bioluminescence is uncertain. The luciferase activity of particulate matter is increased several times by solubilization using 2-mercaptoethanol, which yields soluble luciferase oligomers, such as luciferases A, B, and C.

The total luciferase activity existing in one gram of the dome mesoglea, lappet, and ovary was approximately 100 LU, 1000 LU, and 7 × 10⁵ LU, respectively. Taking account of the quantity of tissue in each organ in the body, these figures suggest that the amount of luciferase stored in the ovary is more than 100 times the total amount of luciferase L in the whole body of a female medusa. The facts that the luciferase is complexed in a stepwise fashion

(dimer, tetramer, etc.) and that these oligomers occur in discrete subcellular particles suggest that the luciferase is being stored for later use. In the case of the male medusae, an insoluble aggregated form of luciferase was not found in the testes, but we are unable to conclude that such a luciferase is absent until all other internal organs have been tested.

### Luciferase in the eggs and during early development

In the eggs, the particulates containing aggregated luciferase are in the cortical layer (Flood *et al.*, 1996). The total content of luciferase in one egg is extremely large for its small size (1 $\mu$g or 5 × 10⁻¹¹ mole/egg; calculated from the data in Table 2), and the luciferase is mostly the aggregated form. Unexpectedly, the eggs contained a negligibly small amount of coelenterazine (1 × 10⁻¹⁴ mole/egg), but some coelenterazine may have been spent by the luminescence reaction that occurs during the preparation of the material.

Unlike most medusae, *Periphylla periphylla* develops directly from egg to medusa without an intermediary, sessile polyp stage (Martinussen *et al.*, 1997; Jarms *et al.*, 1999). The data of Table 2 suggest that the amount of luciferase in the eggs decreases drastically upon fertilization, reaching a minimum at a late embryonic or early juvenile stage (about 3% of the initial amount). Therefore, the biosynthesis of luciferase must start at a later stage of development, because large adult specimens contain large amounts of luciferase. We may see the first sign of such biosynthesis in juveniles with a dome diameter of 8-10 mm. During these juvenile stages, we also see the first differentiation of exumbrellar epithelial photocytes with basically the same organization
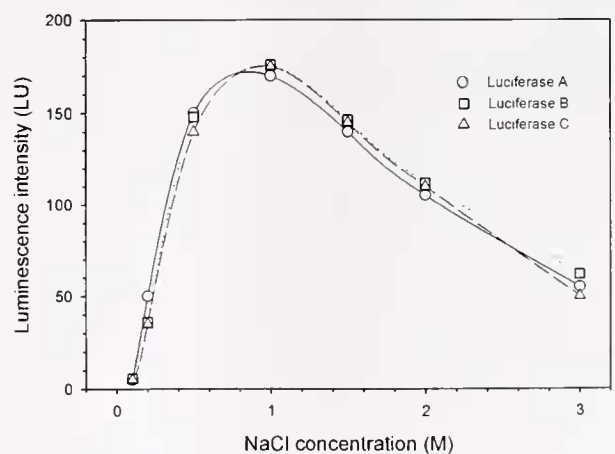


**Figure 7.** Effect of salt concentration on the luminescence intensity of coelenterazine catalyzed by luciferases A, B, and C. The measurements were done in 20 m$M$ Tris-HCl buffer (pH 7.8) containing various concentrations of NaCl, 0.05% BSA, and 10 $\mu$l of 0.1 m$M$ methanolic coelenterazine. With NaCl concentration lower than 0.2 $M$, the intensity gradually decreased by the inactivation of luciferase. For the measurement of each point, 170 LU of each luciferase was used.
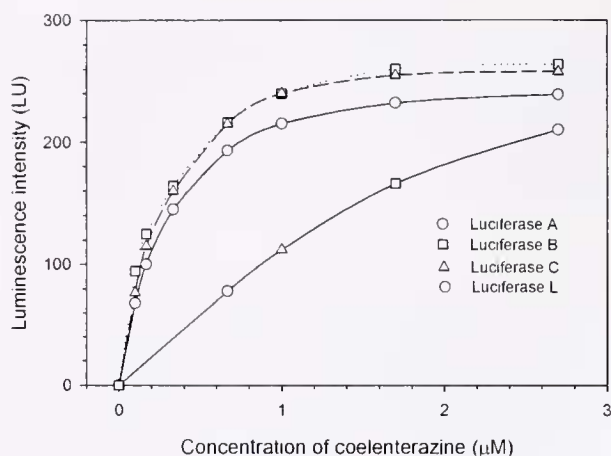
**Figure 8.** Effect of coelenterazine concentration on the luminescence catalyzed by luciferases A, B, C, and L. The measurements were done in the standard buffer. The amounts of sample used were the same as in Figure 6 for luciferases A. B, and C. The data for luciferase L were taken from the previous report (Shimomura and Flood, 1998).

as those found in the adult medusae (Flood, unpubl. obs.). The decrease of luciferase in the eggs, described above, is puzzling and intriguing. Why does the egg contain a large amount of luciferase in the first place? What is the function or purpose of this luciferase?

A similar phenomenon has been observed in the eggs of bioluminescent hydrozoan medusas that contain a $Ca^{2+}$-sensive photoprotein, a complex of oxygenated coelenterazine and an enzyme. In those eggs, the amount of photoprotein slowly declines during the development of the planula larva, and then markedly declines when the planula undergoes metamorphosis to become a polyp (Freeman and Ridgway, 1987).

### Properties of luciferases A, B, and C

The present results indicate that luciferases B (40 kDa) and C (80 kDa) are the dimer and tetramer, respectively, of

the luciferase A monomer (20 kDa). The specific luminescence activities of luciferases A, B, and C were in a range of $1.2{\sim}4.1 \times 10^{16}$ photons/s, showing a tendency to increase slightly as the oligomer size increases. This is the highest specific activity ever reported for a luciferase whose substrate is coelenterazine; the highest in the past was that of the luciferase of the deep-sea shrimp *Oplophorus* ($1.75 \times 10^{15}$ photons/s) (Shimomura *et al.*, 1978).

The specific activity of purified luciferases A, B, and C can vary in a complex manner, depending upon the method of purification and the history of handling; and an increase in the purity is sometimes accompanied by a decrease in the specific activity. Furthermore, the dilution of a solution containing high concentrations of ammonium sulfate always causes a marked decrease in the activity, whereas the addition of 1-2 $M$ guanidine hydrochloride or 0.01% cationic detergent (such as LCC or hexadecyltrimethylammonium bromide) to a luciferase solution often results in an increase in activity. On the other hand, luciferases A, B, and C are highly resistant to inactivation by heat, extreme pHs, and denaturants, such as 4 $M$ guanidine hydrochloride, detergents, and organic solvents. Thus, luciferase A, B, and C are distinctly different from all previously known coelenterazine luciferases that are easily inactivated. The facts noted here may suggest that *Periphylla* luciferase has a unique and unusual tertiary structure, and that the luciferases isolated by us are mixtures of two or more molecular species having conformationally different structures (having different activities) that are not easily separable by chromatography.

The luminescence quantum yields of coelenterazine in the presence of luciferases A, B, and C were close to 0.30 at 24 °C, one of the highest values among coelenterazine luciferases. The quantum yields for other coelenterazine luciferases are *Oplophorus* luciferase, 0.34 at 22 °C (Shimomura *et al.*, 1978), and *Renilla* (sea pansy) luciferase, 0.11 at 23 °C (Inouye and Shimomura, 1997). The high

**Table 2**

*Average luciferase content in single specimens of eggs, embryos, and juveniles*

| Sample | Before 2-ME treatment | | After 2-ME treatment | |
|---|---|---|---|---|
| | Soluble (Luciferase L) | Total | Soluble (Luciferase A, B, C) | Total (mean $\pm$ SD) |
| Egg, dissected from ovary (7) | 46 | 4760 | 17,400 | 19,300 $\pm$ 3,000 |
| Egg, liberated[a] (5) | 35 | 1350 | 4,200 | 5,010 $\pm$ 3,500 |
| Early embryo[b] (5) | 18 | 730 | 2,990 | 3,380 $\pm$ 1,750 |
| Late embryo[c] (3) | 38 | 173 | 620 | 603 $\pm$ 50 |
| Juvenile, 4-5 mm[d] (6) | 16 | 205 | 460 | 490 $\pm$ 431 |
| Juvenile, 8-10 mm[d] (5) | 162 | 370 | 784 | 824 $\pm$ 375 |

Luciferase activity was assayed before and after treatment with 25 m$M$ 2-mercaptoethanol (2-ME) and shown in light units (LU). The number of samples tested is shown in parentheses.

[a] Collected by plankton net. [b] Yolky throughout, with minute grooves for later development of lappets and tentacles. [c] Trace of pigmentation around mouth, still with yolk in stomach. [d] Dome diameter.

efficiency of *Periphylla* bioluminescence suggests that luciferase A might be useful as a highly sensitive reporter.

*The nature of luciferase L*

The specific luminescence activity of luciferase L previously reported ($8 \times 10^{13}$ photons/s) is more than two orders of magnitude lower than that of luciferases A, B, or C (more than $10^{16}$ photons/s), raising doubts about the purity of the luciferase L sample previously reported (Shimomura and Flood, 1998). SDS-PAGE analysis of a sample of luciferase L (Fig. 3) showed that the sample contained only a trace of luciferase A (shown as a weak 24-kDa band) and a large amount of other proteins (an intense broad band of 28-34 kDa), indicating that the purity of the sample was indeed very low, probably about 1%. Such a condition could have arisen from the extremely small amount of luciferase L, as well as from the difference in the initial extracts: luciferase L was purified from an extract containing all soluble proteins, whereas luciferase A, B, and C were purified only from the proteins that were solubilized by 2-mercaptoethanol.

A pure sample of luciferase L (32 kDa), if available, could not, on the basis of its molecular weight, be a simple oligomer of the luciferase A monomer (20 kDa) notwithstanding that it yielded luciferase A by treatment with 2-mercaptoethanol. Thus, luciferase L must be a complex of the luciferase A plus another protein (about 12 kDa). The presence of this accessory protein was confirmed by SDS-PAGE as a band corresponding to 14 kDa (Fig. 3). One of the functions of the accessory protein, apparently, is to solubilize the luciferase, because luciferase L is the only naturally soluble form of luciferase existing in *Periphylla*. The accessory protein, however, has other important functions as judged from the data presently obtained. One of these functions pertains to thermal properties, modifying the temperature-luminescence intensity curve to emit the strongest luminescence at an unusually low temperature, near 0 °C (Fig. 4), and significantly increasing the stability of luciferase activity at high temperatures (Fig. 5). The adaptation of a low-temperature luminescence system as a means of enhancing light emission is understandable for an organism that lives in the deep sea at 3 to 7 °C, but it is puzzling that the accessory protein makes the luciferase L heat-stable to such an unusual level that it withstands even boiling.

Recently, an accessory protein was also found in the luciferase of *Oplophorus* (Inouye *et al.*, 2000). The native form of this luciferase (about 106 kDa) was found to be a complex of two proteins, one 19 kDa and the other 35 kDa. The luciferase function was found in the 19-kDa protein, whereas the role of the 35-kDa accessory protein remains unknown.

The quantum yield of coelenterazine in the presence of luciferase L was previously reported as 0.14 (Shimomura and Flood, 1998), in contrast to the value of about 0.30 for luciferases A, B, or C. The quantum yield value of luciferase L should be reexamined because it was obtained by an unconventional method that is certainly affected by the impurities that destroy coelenterazine and decreases quantum yield. With a pure preparation of luciferase L, the specific activity and the quantum yield are probably close to those of luciferases A, B, and C. Uncertainties concerning luciferase L and the role of the accessory protein will not be completely clarified until pure preparations of luciferase L become available.

## Acknowledgments

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